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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	09/425,075
Filing Date	October 21, 1999
First Named Inventor	CHOUDARY, PRABHAKARA V.
Group Art Unit	1642
Examiner Name	HELMS, LARRY RONALD
Total Number of Pages in This Submission: 20	Attorney Docket Number: UCAL-269

ENCLOSURES (check all that apply)

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment / Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Documents
- ☐ Response to Missing Parts/ Incomplete Application
  - ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Assignment Papers *(for an Application)*
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s
- ☐ After Allowance Communication to Group
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- ☐ Proprietary Information
- ☒ **Communication**
- ☐ Other Enclosure(s) *(please identify below):*

Exhibits for Oral Hearing (13 pgs.)

Exhibit – Article Holliger (5 pgs.)

Postcard

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Signing Attorney/Agent (Reg. No.)	CAROL L. FRANCIS, 36,513 BOZICEVIC, FIELD & FRANCIS, LLP
Signature	
Date	February 11, 2005

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COMMUNICATION

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Customer Number	24353
Atty. Docket No.	UCAL-269
Application Number	09/425,075
Confirmation Number	9044
Filing Date	October 21, 1999
First Named Inventor	CHOUDARY, PRABHAKARA V.
Examiner Name	HELMS, LARRY RONALD
Group Art Unit	1642

Sir:

Please find enclosed a copy of the Exhibits for Oral Hearing and a copy of Holliger (Methods in Mol Biol. 2002 178:348-357) as provided to the Board of Appeals and Interferences during the Oral Hearing on February 10, 2005.

Appellant also brings to the Office's attention an error in footnote 3, page 10 of the Reply Brief filed July 2, 2004. The above-referenced application has, in fact, published on April 27, 2000 as PCT Publication No. WO 00/23579. However, as explained at the Oral Hearing, this fact does not substantially affect the analysis of the Holliger reference in that Holliger clearly teaches away from the claimed invention.

No fees are believed due in connection with this Communication. However, should appellant be incorrect and such fees are due, the Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, to Deposit Account No. 50-0815, order number UCAL-269.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: Feb 11, 2005

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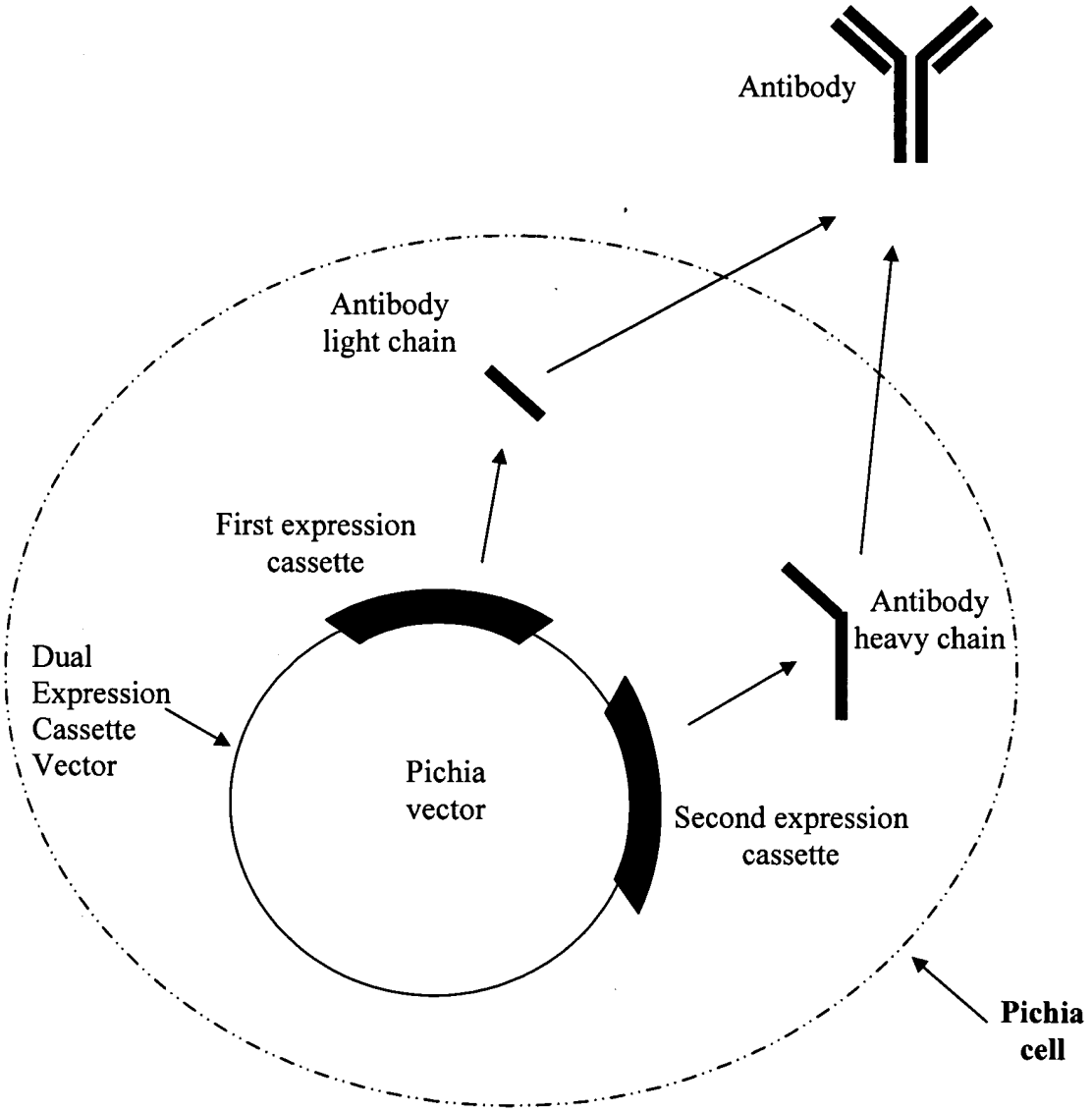
EXHIBITS FOR ORAL HEARING February 10, 2005 APPEAL NO: 2004-2134	Attorney Docket	UCAL-269
	Appellant	Prabhakara V. Choudary
	Application Number	09/425,075
	Filing Date	October 21, 1999
	Group Art Unit	1642
	Examiner Name	Larry Ronald Helms
	Title	*Functionally assembled antigen-specific intact recombinant antibody and a method for production thereof*

SCHEMATIC OF THE CLAIMED INVENTION



Exemplary Claims on Appeal

36. A method for production of an antibody that specifically binds an antigen of interest, the method comprising the steps of:

culturing a recombinant *Pichia* cell, the cell comprising a vector comprising a first and a second expression cassette, wherein:

said first expression cassette comprises a first promoter operably linked to a nucleic acid encoding an immunoglobulin light chain operably linked to a first signal peptide;

said second expression cassette comprises a second promoter operably linked to a nucleic acid encoding an immunoglobulin heavy chain operably linked to a second signal peptide,

and said culturing provides for expression of the immunoglobulin light and heavy chains; and

harvesting specific antigen-binding antibody from culture supernatant, which antibody specifically binds an antigen of interest.

47. A *Pichia* expression vector comprising:

a first and a second expression cassette, said first cassette comprising a first promoter operably linked to a nucleic acid encoding an immunoglobulin light chain operably linked to a first signal peptide, and said second cassette comprising a second promoter operably linked to a nucleic acid encoding an immunoglobulin heavy chain operably linked to a second signal peptide,

wherein introduction of said vector into a *Pichia* host cell provides for production of a recombinant immunoglobulin protein that specifically binds an antigen and is secreted by the host cell.

REJECTIONS ON APPEAL

I. WHETHER THE INVENTION AS CLAIMED IN CLAIMS 36-39 AND 42-50 IS OBVIOUS UNDER 35 U.S.C. §103 IN VIEW OF:

A. HORWITZ (PNAS 85:8678-8682, 1988);

B. CREGG (DEVELOPMENTS IN INDUSTRIAL MICROBIOLOGY 29:33-41, 1998);

C. THE INVITROGEN CATALOG (1997) (PUBLISHED 1/97, YEAST EXPRESSION PAGES 14-19 AND MASTER CATALOG AMENDMENT NOTICE FOR pPICZ VECTORS FORM 4/15/96);

AND

D. ROBINSON (USPN 6,204,023)

II. WHETHER THE INVENTION AS CLAIMED IN CLAIMS 36-39 AND 41-50 IS OBVIOUS UNDER 35 U.S.C. §103 IN VIEW OF:

A. HORWITZ (PNAS 85:8678-8682, 1988);

B. CREGG (DEVELOPMENTS IN INDUSTRIAL MICROBIOLOGY 29:33-41, 1998);

C. THE INVITROGEN CATALOG (1997) (PUBLISHED 1/97, YEAST EXPRESSION PAGES 14-19 AND MASTER CATALOG AMENDMENT NOTICE FOR pPICZ VECTORS FORM 4/15/96);

D. ROBINSON (USPN 6,204,023)

AND

E. VANDERLAAN (USPN 5,429,925)

SUMMARY OF THE CITED ART

The references that are the basis for the §103(a) rejection are cited for their disclosures are follows:

HORWITZ

– ***single expression cassette vector*** system for production of functional antibodies in ***Saccharomyces cerevisiae***.

CREGG

-- ***Pichia alcohol oxidase promoter***.

THE INVITROGEN CATALOG

-- ***single expression cassette vector*** system for ***Pichia***

VANDERLAAN

-- ***anti-dioxin antibody***.

ROBINSON

-- ***dual expression cassette vector*** system for producing functional antibodies in ***mammalian*** cells

-- use of ***single expression cassette vector*** to produce functional antibody ***in Saccharomyces cerevisiae ("yeast")***

-- asserted suggestion that a ***dual expression cassette*** system could be used for antibody production in ***"yeast"***

Robinson is the critical reference for both rejections on appeal.

Robinson assertedly provides

1) dual expression cassette vectors and

2) a suggestion to use dual expression vectors in "yeast"

SUMMARY OF APPELLANT'S POSITION

- The *prima facie* case of obviousness has not been made for at least the following reasons:
  - The assertion that Robinson provides a suggestion to use a dual expression cassette vector in *Pichia* is based on an incorrect and improper interpretation of the term "yeast" in Robinson
  - Regardless of what "yeast" means, is there is no reasonable expectation of success in using a *dual expression cassette* in *Pichia* to successfully produce a functional antibody.
  - Regardless of what "yeast" means, the art, using no uncertain terms, teaches away from the claimed invention.

- Appellant's position is supported by facts.

- Appellant's analysis of the facts and position are supported by a declaration by James B. Trager, Ph.D., an expert in the field of yeast molecular biology who is an uninterested declarant.

- The Examiner has never provided factual evidence to rebut Appellant's position.

- The facts on the record have not been given their full evidentiary weight as a whole.

SUMMARY OF APPELLANT'S POSITION (con'd)

1) **IN ORDER TO ESTABLISH THE EXAMINER ARGUES THAT ROBINSON SUGGESTS THE CLAIMED INVENTION BECAUSE ROBINSON STATES:**

> The following approaches can be taken to simultaneously express both light and heavy chain genes in yeast.
> (1) The light and heavy chain genes are each attached to a yeast promoter and a terminator sequence and placed on the same plasmid. This plasmid can be designed for either autonomous replication in yeast or integration at specific sites in the yeast chromosome.

Robinson col. 15, lines 13-20.

The suggestion to use a *dual* expression cassette vector to express an *antibody* in *Pichia* is not provided by any of the other cited prior art references, thus the teachings of Robinson are critical to this rejection.

One of skill in the art, the term "yeast" can either mean either:

a) a particular genus of fungi that encompasses ***over 25,000 different genera and species of yeast***, which broad genus would include Pichia, or

b) a single species of fungi, *Saccharomyces cerevisiae*.

The Examiner's Position: "yeast" in Robinson is a generic term that encompasses Pichia, thus providing the suggestion to use a dual expression cassette vector in *Pichia*.

Appellant's Position: The term "yeast", as used in Robinson, means "*Saccharomyces cerevisiae*". If this is the case, then there is no suggestion in Robinson to use a dual expression cassette in *Pichia*.

FACT:

The context of Robinson's use of the term "yeast" demonstrates that the term "yeast" as used in Robinson, means "*Saccharomyces cerevisiae*".

SUMMARY OF APPELLANT'S POSITION (con'd)

FACT:

The context of Robinson's use of the term "yeast" demonstrates that the term "yeast" as used in Robinson, means "*Saccharomyces cerevisiae*".

Supporting evidence for this statement is found throughout Robinson's disclosure, particularly at the following:

Robinson refers to the *S. cerevisiae* gene as "the yeast invertase gene", (Robinson, col.44, lines 40-47; emphasis added)

> Yeast cells are capable of recognizing mammalian secretion signal sequences and of directing secretion of mammalian proteins (Hitzman et al., supra). There is, however, evidence which suggests that certain native yeast signal sequences are more effective than mammalian signal sequences at directing secretion of some mammalian proteins from yeast (Smith et al., *Science* 229:1219 (1985)). One example is the signal sequence for the yeast invertase gene. To improve the efficiency of light and heavy chain

Other examples:

-- Robinson refers to the *S. cerevisiae* PGK promoter as "the yeast PGK promoter", (Robinson, col. 9, lines 41-50; emphasis added).
-- Robinson refers to the origin of replication of the 2-micron plasmid endogenous to *S. cerevisiae as* "the yeast origin of replication, oriY, a cis-acting sequence (REP3) from the yeast endogenous 2-micron plasmid." (Robinson, col. 45, line 59-col 46, line 4; emphasis added).

FACT:

At no point in the disclosure does Robinson define "yeast" as anything other than *S. cerevisiae,* and never uses the term "yeast" to describe anything other than *S. cerevisiae*.

SUMMARY OF APPELLANT'S POSITION (con'd)

Appellant's analysis of Robinson is supported by Dr. Trager's analysis:

> 11. As is known by the Skilled Person, the word "yeast" has one of two meanings, depending on the context of how it is used. In the first meaning, "yeast" solely refers to the species of *Saccharomyces cerevisiae*, commonly known as "brewer's yeast". For example, if a Skilled Person says he works in a "yeast lab", he is indicating that he works in a lab that works on *S. cerevisiae*. In the second meaning, "yeast" refers to a genus of fungi that encompasses over 25,000 species from the following families *Saccharomyes, Pichia, Candida, Schizosaccharomyces, Neurospora*, and others. As an example, throughout this declaration I have used the word "yeast" in its second meaning, referring to a genus of fungi. In other words, depending on the context of how the word "yeast" is used in a reference, it refers to either *S. cerevisiae*, or a genus of over 25,000 species of fungi.
>
> 12. From the context of how the word "yeast" is used in Robinson, a Skilled Person would recognize that Robinson uses the word yeast with its first meaning, as a reference to *S. cerevisiae*. A Skilled Person would recognize this because Robinson uses the terms, "yeast" and "*S. cerevisiae*" interchangeably. For example, Robinson refers to the *S. cerevisiae* gene as "the yeast invertase gene", refers to the *S. cerevisiae* PGK promoter as "the yeast PGK promoter", and refers to the origin of replication of the 2-micron plasmid endogenous to *S. cerevisiae* as "the yeast origin of replication, oriY, a cis-acting sequence (REP3) from the yeast endogenous 2-micron plasmid." At no point in the disclosure does Robinson suggest that "yeast" encompasses anything other than *S. cerevisiae*.

Trager declaration, ¶P 11 and 12 (emphasis added).

CONCLUSION: The term "yeast" in Robinson has been incorrectly and improperly construed to mean a generic term encompassing a genus of fungi encompassing *Pichia*. *Instead,* Robinson at best merely suggests a dual expression cassette for expressing antibodies ***S. cerevisiae***.

This suggestion is not provided by any of the other cited prior art references, and thus the rejection is improperly established.

SUMMARY OF APPELLANT'S POSITION (con'd)

2) REASONABLE EXPECTATION OF SUCCESS:

-- REGARDLESS OF WHAT "YEAST" MEANS, IS THERE IS NO REASONABLE EXPECTATION OF SUCCESS IN USING A *DUAL EXPRESSION CASSETTE* IN *PICHIA* TO SUCCESSFULLY PRODUCE AN ANTIBODY.

FACT:

The common knowledge of the ordinarily skilled artisan would lead him to believe the claimed invention would not be expected to work

In particular, Dr Trager declared:

> 17. The Invention involves a dual expression cassette vector for expression of immunoglobulin heavy and light chains of an antibody. Because two expression cassettes are on the same vector, a Skilled Person, would not have any reasonable expectation of success in making and using such a vector because of the problems associated with intra-molecular recombination (*e.g.* occurring when two parts of a vector are similar in nucleotide sequence), transcriptional interference (*e.g.* occurring when transcription of one expression cassette does not terminate properly, so that transcription "reads through" so as to interfere with transcription of the second expression cassette), and translational interference (*e.g.* occurring when transcriptional read-through of the first expression cassette produces an antisense molecule that interferes with the translation of the RNA from the second expression cassette). Such problems are commonly associated with such dual expression cassette vectors, especially when the expression cassettes contain polynucleotides with similar or identical sequences (for example similar promoters, signal sequence-encoding polynucleotides or terminators). The usual way of making vectors usually involves an intermediate vector production step in bacteria (*e.g., E. coli*) these problems would pose serious technical barriers. Thus, even if these problems only happened in bacteria, they would still impact the question of whether or not a Skilled Person would make and use such a vector.

(see also Trager declaration ¶¶ 15-16)

SUMMARY OF APPELLANT'S POSITION (con'd)

3) TEACHING AWAY:

-- REGARDLESS OF WHAT "YEAST" MEANS, THE ART, IN NO UNCERTAIN TERMS, TEACHES AWAY FROM THE CLAIMED INVENTION.

Holliger (*Methods in Mol Biol.* 2002 178:348-357) reviews antibody expression in *Pichia.* –

FACT: Holliger teaches away from use of a dual expression cassette vector
Holliger, p. 351 (emphasis added).

Ab Fragment Expression in P. pastoris 351

5. Sterile Millipore H_2O.
6. 1 *M* sorbitol (SORB).
7. *Pichia* expression vectors: pPIC (AOX promoter) or pGAPZ (Invitrogen) (*see* Note 4). Both vectors have a C-terminal c-myc epitope tag for convenient immunodetection with an anti-myc Ab (9E10) (Invitrogen), as well as a C-terminal hexahistidine tag for immobilized metal-affinity chromatography (IMAC) purification.
8. Ab clone. Because bicistronic expression works only poorly in *Pichia* (unlike *Escherichia coli*), it is preferable to use single-chain Ab formats (e.g., scFv, diabody). Two-chain Ab formats (e.g., Fvs, Fabs, bispecific diabodies) require that the two chains be cloned and transformed separately.

Dr. Trager declared Holliger discourages use of dual expression cassette vectors in *Pichia.* (see next page)

Examiner: (Examiner's Answer, pg. 11) – dismissed Holliger because "would not have been available to the skilled artisan at the time of the invention and the argument is moot"

Rebuttal:

Holliger is a **review article** and **cites publications ONLY from 1999 – 2000**

The instant application was filed in 1999 with a 1998 priority date

Holliger reviews the art relevant to the time of the invention

See also – *Ex parte Erlich*, 22 USPQ 1462 (Bd. Pat. App. & Inter. 1992) (MPEP §2124) – (references which do not qualify as prior art because they postdate the claimed invention may be relied upon to show the level of ordinary skill in the art at or around the time the invention was made)

SUMMARY OF APPELLANT'S POSITION (con'd)

3) TEACHING AWAY:

Dr Trager agrees with the analysis of Holliger:

21. The second of these references, Holliger (*Methods in Mol Biol.* 2002 178:348-357; Exhibit B), states, in point 8 on page 351 "Because bicistronic expression works only poorly in *Pichia* (unlike *E. coli*), it is preferable to use single-chain Ab formats. Two chain Ab formats require that the two chains be cloned and transformed separately". (Underlining added). Hollinger, therefore, appears to say that single expression cassette vectors are **required** if expression of two different chains of an antibody is desired.

22. Based on the foregoing discussion, it is my unequivocal opinion that a Skilled Person, in view of the cited publications (i.e., Robinson et al, *etc.*), would not find the invention obvious because the literature and common knowledge in the field would lead them away from doing so. Given that two different reviews of the field of antibody expression in *Pichia* categorically and in no uncertainty direct away from using dual expression cassette vectors, why would a Skilled Person expect it would work?

CONCLUSION

For at least the reasons summarized here, as well as those further articulated in the Appeal Brief, the rejections of the claims under 35 U.S.C. §103(a) should be withdrawn.

10. The STE solution used to render the bacterial periplasm hypertonic typically contains little rFab after the bacteria are pelleted. However, rFab leakage may occur if the bacteria are incubated in STE for more than 1 h. Therefore, do not incubate the bacteria in STE for more than 45 min on ice.
11. After centrifugation, care should be taken not to pour the cells out of the bottle because the pellet is loose after incubation in STE. It may be necessary to respin the bottles to form appropriate conditions for good pellet formation.
12. By comparing Western blotting results of crude rFab periplasmic extracts, we observed that the total amount of rFab reactive to anti-mouse Fab Ab was not always detected by the Ni-NTA conjugate. This suggests that the His tag fused to the HC can be proteolytically cleaved within the bacterial periplasm. Therefore, the successful purification of rFabs should be checked after metal chelate chromatography and before further purification. The addition of protease inhibitors to the periplasmic extract may help reduce proteolysis.
13. Concentrated rFab preparations may be subjected to size-exclusion chromatography to remove residual impurities, such as aggregated rFabs and contaminating *E. coli* proteins, which can co-elute from the His-bind column. This may increase the avidity of the rFab preparation.

References

1. Barbas, C. F., Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. USA* **88**, 7978–7982.
2. Raffaï, R., Vukmirica, J., Weisgraber, K. H., Rassart, E., Innerarity, T. L., and Milne, R. (1999) Bacterial expression and purification of the Fab fragment of a monoclonal antibody specific for the low density lipoprotein receptor-binding site of human apolipoprotein E. *Protein Exp. Purif.* **16**, 84–90.
3. Huse, W. D., Sastry, L., Iverson, S. A., Kang, A. S., Alting-Mees, M., Burton, D. R., Benkovic, S. J., and Lerner, R. A. (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **246**, 1275–1281.
4. Bothmann, H. and Plückthun, A. (1998) Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nature Biotechnol.* **16**, 376–380.
5. Kipriyanov, S. M., Moldenhauer, G., and Little, M. (1997) High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *J. Immunol. Methods* **200**, 69–77.
6. Raffaï, R., Weisgraber, K. H., MacKenzie, R., Rupp, B., Rassart, E., Hirama, T., Innerarity, T. L., and Milne, R. (2000) Binding of an antibody mimetic of the human low density lipoprotein receptor to apolipoprotein E is governed through electrostatic forces. *J. Biol. Chem.* **275**, 7109–7116.
7. Forsberg, G., Forsgren, M., Jaki, M., Norin, M., Sterky, C., Enhörning, Å., et al. (1997) Identification of framework residues in a secreted recombinant antibody fragment that control production level and localization in *Escherichia coli*. *J. Biol. Chem.* **272**, 12,430–12,436

31

Expression of Antibody Fragments in *Pichia pastoris*

Philipp Holliger

1. Introduction

Since the advent of hybridoma technology 25 years ago, monoclonal antibodies (Abs) have revolutionized many aspects of biological research and health care. After some initial setbacks, Abs are also beginning to make an impact as therapeutic agents in the clinic *(1)*. In the last decade, novel selection technologies, such as phage display and ribosome display, have emerged, allowing the isolation of Abs directly from diverse repertoires of V genes *(2)*. Phage display, in particular, has become a mature technology, allowing Abs with nanomolar (or even subnanomolar) affinities to be made to order against virtually any Ag, including self Ags (*3*; *see* Note 1). Furthermore, using high-throughput technologies, such as robotics and array screening, a multitude of Abs against a given Ag (or mixtures thereof) can now be isolated simultaneously, greatly increasing the options for assay or drug development (*see* Note 2).

Regardless of the method of isolation, Abs have to be expressed in recombinant form for screening, characterization, and application. Although both whole Abs and Ab fragments (Fabs *[4]*, Fvs *[5]*, scFvs *[6]*, and diabodies *[7]*) can be expressed in eukaryotic cells (e.g., mammalian yeast, plant, and insect cells *[8]*), it is time-consuming and cost-intensive. Expression in bacteria, particularly secretion to the bacterial periplasm (*see* Note 3), is a quick and cheap alternative and is best-suited for the screening and characterization of a large number of Ab variants. Some phagemid vectors *(9)* even offer a built-in switch between phage display and soluble expression, allowing direct screening of Ab fragments isolated by phage selection without the need for

From: *Methods in Molecular Biology, vol. 178: Antibody Phage Display: Methods and Protocols*
Edited by: P. M. O'Brien and R. Aitken © Humana Press Inc., Totowa, NJ

recloning. However, because of the lack of glycosylation, only Ab fragments and not whole Abs (*see* above) can be produced in a functional form in bacteria.

Expression yields in bacteria can vary widely between different Ab fragments, but yields of 1–10 mg/L are typical for shaker-flask cultures. Using fermentation technology, expression levels of up to 1 g/L can be reached *(10)*. Generally, expression yields are a function of the Ab fragment sequence and format (e.g., Fv vs Fab), rather than the expression system. Fvs (5–50 mg/L) often give the highest expression yields (but are sometimes unstable), followed by scFvs, then diabodies, with Fabs usually giving the lowest expression yields (0.1–1 mg/L). As a rule of thumb, Ab fragments derived from phage libraries tend to give higher yields than those recloned from hybridomas. However, some Ab fragments are generally difficult to express in *Escherichia coli*. Although yields of difficult fragments can sometimes be improved through protein engineering *(11,12)* or selection *(12)*, no general rules have emerged.

A pragmatic alternative to time-intensive optimization of bacterial expression is the use of a eukaryotic expression host. The methylotrophic yeast, *Pichia pastoris*, combines some of the advantages of eukaryotic expression systems, e.g., more efficient folding of multidomain and cys-rich proteins, with the speed and cost efficiency approaching that of prokaryotic systems *(13)*. Optimal expression in *Pichia* is dependent on a range of factors, including codon usage *(14)*, aeration, temperature control (at 28–30°C: *Pichia* is temperature-sensitive) and methanol (MeOH) concentration (when using the alcohol oxidase 1 [AOX1] promoter). Protease-sensitive proteins are usually not well expressed, because *Pichia* secretes a number of proteases. Nevertheless, *Pichia* has become a popular host for heterologous protein expression *(13)*, and a range of Ab fragments, including scFvs and diabodies, have been successfully expressed in *Pichia*, with yields up to 200 mg/L *(15)* in shaker flasks and >1 g/L in fermentor cultures.

This chapter focuses on the expression of functional Ab fragments by the yeast, *P. pastoris (3)*. Using appropriate expression vectors, the Abs are secreted into the yeast culture supernatant, and purified using affinity chromatography. The Ag specificity and binding affinity of the Abs can be determined using BIAcore technology or other suitable methods.

2. Materials

1. *P. pastoris* strain, GS115 (Invitrogen) (*see* Note 4).
2. YP medium: 1% (w/v) yeast extract, 2% peptone.
3. YPD medium: 1% yeast extract, 2% peptone, 2% glucose.
4. YPDS medium: 1% yeast extract, 2% peptone, 2% glucose, 1 *M* sorbitol. For YPDS plates, add 2% (w/v) agar.

5. Sterile Millipore H_2O.
6. 1 *M* sorbitol (SORB).
7. *Pichia* expression vectors: pPIC (AOX promoter) or pGAPZ (Invitrogen) (*see* Note 4). Both vectors have a C-terminal c-myc epitope tag for convenient immunodetection with an anti-myc Ab (9E10) (Invitrogen), as well as a C-terminal hexahistidine tag for immobilized metal-affinity chromatography (IMAC) purification.
8. Ab clone. Because bicistronic expression works only poorly in *Pichia* (unlike *Escherichia coli*), it is preferable to use single-chain Ab formats (e.g., scFv, diabody). Two-chain Ab formats (e.g., Fvs, Fabs, bispecific diabodies) require that the two chains be cloned and transformed separately.
9. Appropriate restriction enzymes and DNA purification and other reagents for molecular cloning of Ab sequences.
10. *E. coli* strain for propagation of plasmid vectors, e.g., TG1.
11. Zeocin (Invitrogen): stock solution 100 mg/mL. Store at –20°C (in the dark).
12. 2TY medium, supplemented with 0.1–5% (w/v) glucose. Autoclave for sterilization, then supplement with sterile-filtered (0.2 µm) glucose (20%).
13. TYE agar (for plates), supplemented with 0.1–5% (w/v) glucose. Autoclave, then supplement with sterile-filtered glucose (20%).
14. TE: 10 m*M* Tris-HCl, 1 m*M* ethylyenediamine tetraacetic acid (EDTA), pH 8.0. Filter-sterilize.
15. Electroporator, e.g., Bio-Rad Genepulser.
16. Methanol.
17. 1 *M* Phosphate buffer: 132 mL 1 *M* KH_2HPO_4, 868 mL 1 *M* KH_2PO_4. Adjust pH to 6.0 with KOH. Filter-sterilize.
18. 10X YNB: 134 g yeast nitrogen base (with NH_4SO_4)/L MilliQ H_2O. Autoclave.
19. 500X B: 20 mg biotin/100 mL MilliQ H_2O. Filter-sterilize.
20. 10X GY: 10% glycerol (v/v) in MilliQ H_2O. Filter-sterilize.
21. BMGY: 100 mL 1 *M* phosphate buffer, pH 6.0, 100 mL 10X YNB, 2 mL 500X B, 100 mL 10X GY in 1 L of YP medium. Filter-sterilize.
22. BMMY: as BMGY, but replace the 10X GY with 100 mL 5% MeOH (v/v) in H_2O. Filter-sterilize.
23. BIAcore machine and software, CM5 BIAcore chip.
24. N-ethyl-Ni-(diaminopropyl) carbodimide (EDC); (*N*-hydroxysuccimide (NHS).
25. Ag of interest, purified.
26. 100 m*M* Na acetate, pH 6.0–4.0; 1 *M* ethanolamine.
27. Phosphate buffered saline (PBS).
28. Ni-NTA resin (Qiagen).
29. IMAC phosphate buffer: 29.82 g NaH_2PO_4, 5.52 g $NaH_2PO_4 \cdot H_2O$, 147 g NaCl/L. Adjust the pH to 7.5 with 1 *M* NaOH.
30. Imidazole (Sigma).
31. IMAC Loading buffer: 50 mM IMAC phosphate buffer, pH 7.5, 0.5 *M* NaCl, 20 m*M* imidazole. Dilute IMAC phosphate buffer fivefold in H_2O, then add imidazole powder to give a final concentration of 20 m*M*. Store at 4°C.

3. Methods

3.1. Preparation of Electrocompetent Pichia GS115

1. Inoculate a single colony of *Pichia* GS115 into 5 mL YPD medium and grow overnight at 30°C.
2. Dilute the overnight culture 1:1000 into fresh YPD medium (e.g., add 1 mL overnight culture into 1 L) and grow overnight at 30°C.
3. Pellet the cells at 1500*g* for 20 min at 4°C, then resuspend in an equal volume of ice-cold sterile Millipore H_2O.
4. Pellet the cells, then resuspend in 0.5 vol ice-cold Millipore H_2O.
5. Pellet the cells, then resuspend in 0.2 vol ice-cold sterile 1 *M* SORB.
6. Pellet the cells, then resuspend in 0.005 vol ice-cold sterile 1 *M* SORB.
7. Use the cells for transformation, or store in 0.1 mL aliquots by flash-freezing on dry ice and store at –70°C (*see* Note 5).

3.2. Cloning of Ab Fragments for Expression in P. pastoris

1. Clone the selected Ab fragment(s) into the appropriate *Pichia* expression vector in *E. coli* using standard cloning procedures (*see* Notes 6 and 7).
2. Prepare plasmid DNA from the resulting clones by miniprep procedures, then linearize with *Avr*II (pGAPZα) or *Bst*XI (pPICZ). Extract the digests with phenol:chloroform (1:1) once, and precipitate the DNA with ethanol. Resuspend the precipitated pellet in 5 µL TE.
3. Add 2.5 µL DNA to 50 µL electrocompetent *Pichia* cells and electroporate at 1.5 kV, 25 µF, and 200 Ω. Resuspend the cells in 1 mL 1 *M* SORB and incubate for 2 h at 30°C.
4. Plate the transformed cells on YPDS plates containing 50 µg/mL zeocin and incubate at 30°C. Colonies (10–1000) will appear in 3–4 d.

3.3. Expression of Ab Fragments in Pichia (see Note 8)

3.3.1. Expression in pPIC (MeOH Induction) (see Note 9)

1. Inoculate a colony expressing a pPIC/Ab clone into 1 mL YPD medium containing 0.1mg/mL zeocin and grow overnight at 30°C.
2. Dilute the overnight culture 1:100 into fresh YP medium (e.g., dilute 0.1 mL into 10 mL) and grow for 24 h at 30°C. Add MeOH to a final concentration of 0.5% (v/v) and grow for a further 24 h. Repeat the MeOH addition every 24 h until the culture is harvested after 1–4 d (*see* Note 10).

   Alternatively, dilute the overnight culture 1:100 into fresh BMGY medium (e.g., dilute 0.1 mL into 10 mL) and grow at 30°C to an optical density 600 nm of 4.0. Pellet the cells by centrifugation at 1500*g* for 20 min, then resuspend in an equal volume of BMMY medium and grow for 24 h at 30°C. Add MeOH (0.5% [v/v] final concentration) and grow for a further 24 h. Repeat the MeOH addition every 24 h until the culture is harvested (after 1–4 d).
3. Spin the culture at 10,000*g* for 30 min at 4°C and collect the supernatant (*see* Note 11). The supernatant can be used directly for analysis of Ab expression (e.g. by enzyme-linked immunosorbant assay [ELISA] or BIAcore) or can be stored and/or purified before use (*see* Notes 12 and 13).

3.3.2. Expression in pGAPZ (Constitutive Expression)

1. Inoculate a colony expressing a pGAPZα/Ab clone into 1 mL YPD medium containing 0.1 mg/mL zeocin and grow overnight at 30°C.
2. Dilute the overnight culture 1:100 (e.g., dilute 0.1 mL into 10 mL) into fresh YPD medium (without zeocin) (*see* Note 14). Grow the culture at 30°C for 1–4 d (*see* Note 11).
3. Harvest the culture supernatant and store or purify the Ab as described in Subheading 3.3.1.

3.4. Analysis of Ab Binding by BIAcore (see Note 15)

This procedure can be used to quickly investigate Ab specificity using crude extracts of yeast culture supernatant as an alternative to ELISA (*see* Note 16). If purified material is used, the method can also be used to determine affinity. More information about the BIAcore instrument and the method can be found at the BIAcore website: http://www.biacore.com.

1. Dock a research-grade CM5 chip (BIAcore) in the BIAcore machine, according to the manufacturer's instructions.
2. Amine-couple 500–5000 resonance units (RU) of the desired Ag, according to the manufacturer's instructions (the amount of Ag this corresponds to depends on its molecular weight, because the BIAcore signal [RU] is mass-dependent). Briefly, activate the chip surface with EDC–NHS (typical injection is 30 µL at 10 µL/min flow rate). Inject the Ag (typically, 100 µg/mL in 100 m*M* Na acetate, pH 6.0–4.0) (*see* Note 17). Stop the coupling reaction by injecting 1.0 *M* ethanolamine, which blocks the remaining activated sites.
3. Filter the recombinant Ab samples through a 0.2 µm filter before injection.
4. Pass the Ab solution over the chip surface (typical injection times range from 1 to 10 min at flow rates of 5–50 µL/min). An increase in RU indicates binding.
5. Plot a graph of RU vs time. Analyze the binding affinity and/or kinetics using the BIAcore software.

3.5. Purification of Recombinant Ab Fragments by IMAC

Like Ab fragments expressed from polyhistidine-tagged *E. coli* expression vectors, Abs expressed in *P. pastoris* using the pPIC or pGAPZ plasmids can be purified by IMAC. The Ab-containing culture supernatants must first be dialyzed against PBS before purification to remove chelating compounds present in the growth media (*see* Note 18).

1. Dialyze the culture supernatant against two changes of PBS (ideally at 4°C). For smaller volumes, dialysis tubing with a 10 kDa cutoff is suitable. For large

volumes, dialysis is best performed using tangential flow filtration using repeated addition of PBS during the concentration process (*see* Note 12).

2. Add the appropriate amount of Ni-NTA resin to an appropriate column and equilibrate the resin with 10 column volume loading buffer (e.g., for 5 mL resin, use 50 mL buffer). 1 mL Ni-NTA resin is usually sufficient to purify 2–3 mg Ab fragment (*see* Note 19).
3. Load the dialyzed Ab preparation onto the column (either by gravity flow or using a peristaltic pump) and collect the unbound fraction.
4. Wash the column with at least 10 column volume loading buffer. If the washing process can be observed using an UV-flowcell, washing should continue until a stable baseline is reached.
5. Elute the Ab fragments using an imidazole gradient from 35 to 200 m*M* in loading buffer (*see* Note 20). Elution peak fractions should ideally be detected using an UV-flowcell. The elution of Ab should be confirmed by ELISA or BCA protein assay (Pierce).
6. Dialyze the Ab fractions into the desired buffer (e.g., PBS) to remove the imidazole, then concentrate the Ab by ultrafiltration using a stirred cell device with an appropriate cutoff (10 kDa for scFvs, 30 kDa for Fabs, and diabodies).
7. Aliquot the Ab preparations for storage. Concentrated Ab preparations (>0.5 mg/mL) in PBS are suitable for freezing. As a rule, preparations should always be flash-frozen in dry ice or liquid nitrogen and never in a –20°C freezer. Once frozen, a –20°C freezer is suitable for short- to medium-term storage.

4. Notes

1. Protocols for the selection of Ab specificities from phage libraries have been published (*16*) and several libraries are available to researchers (http://www.mrc-cpe.cam.ac.uk/phage/index.html).
2. An alternative method for isolating human Abs is transgenic "human" mice with partial human heavy- and light-chain loci inserted into their genomes (*17*). A possible advantage of this approach may be the ability to use the isolated hybridomas directly for production of whole Abs with no need for further genetic manipulation.
3. Ab fragments can be expressed both intra- and extracellularly, i.e., secreted. Intracellular expression of Ab fragments in *E. coli* usually gives rise to insoluble aggregates (inclusion bodies) that have to be refolded. Secretion from bacteria (to the periplasm) or yeast mimics the natural expression and folding pathway of Abs and often provides a more direct route to functional Ab fragments.
4. *P. pastoris* strains and expression vectors are commercially available from Invitrogen. *Pichia* protocols are available to download from the Invitrogen website (http://www.invitrogen.com/manuals.html).
5. Freezing reduces competence. In order to obtain the highest possible transformation efficiencies, it is advisable to use freshly prepared cells. However, frozen competent cells are perfectly adequate for standard transformations. Before use, thawed frozen cells should be washed once in 0.5 mL ice-cold sterile 1 *M* sorbitol.
6. Ab fragments can be cloned using PCR directly from hybridomas using standard methodology (a kit comprising mouse V-gene-specific primers is available from Pharmacia) or isolated from phage selected from libraries using panning procedures.
7. When using zeocin selection in combination with high-salt media (TYE, 2TY), it is advisable to use a final concentration of 100 µg/mL zeocin for selection. Transformed *E. coli* cells should be incubated for 1–2 h in 2TY, 1% glucose at 37°C, before plating on zeocin plates because zeocin resistance is expressed slowly.
8. There are two types of promoter systems available in *Pichia*; the MeOH-inducible AOX1 promoter and the constitutive glyceraldehyde-phosphate dehydrogenase (GAPDH) promoter. Expression of some proteins can be higher under control of the GAPDH promoter (using glucose as a carbon source) than by MeOH induction of the AOX1 promoter. Both promoters should be tried because expression yields can differ dramatically. Furthermore, expression levels usually vary a great deal among different *Pichia* clones. It is advisable to screen a number of colonies for expression in order to identify high-expressing "jackpot" clones. *Pichia* expression can also depend on good aeration so expression cultures should be grown with vigorous shaking (350 rpm).
9. For optimal protein yields with MeOH induction, the alternative may be more effective than the primary methods.
10. Protein expression takes place over 1–4 d at 30°C. Maximum yields usually are obtained by harvesting on d 2 or 3.
11. Respin the culture if the supernatant is not clear.
12. The cleared supernatant can be used directly in ELISA or BIAcore analysis, or can be stored at –20°C prior to purification. For large-scale preparations (>1 L), it may be advantageous to concentrate the supernatant before purification. Various concentration methods are available (e.g., ammonium sulphate precipitation), but ultrafiltration is preferable. Filter the supernatant through a 16 µm tangential-flow filter (Flowgen Minisette system) with the use of a peristaltic pump to remove small debris. Concentrate the supernatant using the Minisette system, using a tangential-flow filter minisette with an appropriate cutoff (e.g., 10 kDa for scFvs and Fvs, or 30 kDa cutoff for Fabs and diabodies). The concentrate (typically, 0.3–0.5 L) can be stored at –20°C prior to purification.
13. Ab fragments produced in *Pichia* often have nonhomogenous N-termini because of incomplete processing of the leader peptide, giving rise to fuzzy bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. These N-terminal extensions can be shaved off using 5 µg/mL trypsin digestion for 5 min (immobilized TPCK trypsin [Pierce]). The reaction is stopped by addition of Pefabloc trypsin inhibitor (at 5 µg/mL) and removal of the enzyme gel by centrifugation. Because trypsin may also cleave off polyhistidine tags, it is advisable to carry out the digestion after purification.

14. Zeocin selection during expression is unnecessary and can reduce the yield of expressed protein.
15. These methods are not limited to Ab expressed in *Pichia* and can also be used for determining Ag specificity (crude periplasmic extracts) and binding affinity (purified Ab) of Ab expressed in *E. coli*.
16. Either ELISA or BIAcore can be used to determine affinity constants of purified Ab fragments. In my opinion, BIAcore is superior to ELISA-based methods, provided attention is paid to the oligomerization state of the Ab fragment. Multimeric fragments (e.g., some scFvs, bivalent diabodies) bind to solid-phase Ags with much-increased affinity (avidity). Failure to take this into account can lead to an overestimation of affinity by several orders of magnitude. On the other hand, multimerization can be helpful in increasing the sensitivity of Ag-binding assays, particularly for Ab fragments with modest affinities for Ag. For methods relating to Ab multimerization (and expression), *see* **ref. *18***. BIAcore can also be used to measure Ag-binding kinetics.
17. For optimal coupling efficiencies, the pH should be determined by experimental analysis (knowledge of the isoelectric point value of the Ag is not sufficient). Coupling should be spontaneous. For slow-reacting Ags, it may be appropriate to slow down the flow rate.
18. Purification by IMAC has advantages beyond other purification methods because of its versatility and mild elution conditions. The commonly used rich medium for *Pichia* (YP) expression (and for *E. coli* [2TY or Luria-Bertani broth]) contain metal-chelating compounds, which strip the metal from the IMAC column (the same also applies for periplasmic preparations from *E. coli*-containing EDTA). Metal loss from the IMAC column is easy to spot because the column loses its blue-green color (in the case of Ni^{2+}) and turns white.
19. Ab fragments can give widely differing expression yields, ranging from 1 to 100 mg/L of induced *Pichia* culture. It is thus advisable to determine approximate expression levels before embarking on purification.
20. Most Ab fragments elute between 50 and 100 m*M* imidazole. Diabodies and triabodies, which have two and three hexahistidine tags, respectively, usually elute at higher concentrations (50–200 m*M* imidazole).

References

1. Milstein, C. and Waldmann, H. (1999) Optimism after much pessimism: what next? *Curr. Opin. Immunol.* **11**, 589–591.
2. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994) Making antibodies by phage display technology. *Ann. Rev. Immunol.* **12**, 433–455.
3. Griffiths, A. D. and Duncan, A. R. (1998) Strategies for selection of antibodies by phage display. *Curr. Opin. Biotechnol.* **9**, 102–108.
4. Better, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988) *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041–1043.
5. Skerra, A. and Pluckthun, A. (1988) Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* **240**, 1038–1041.

6. Huston, J. S., Levinson, D., Mudgett, H. M., Tai, M. S., Novotny, J., Margolies, M. N., et al. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **85**, 5879–5883.
7. Holliger, P., Prospero, T., and Winter, G. (1993) "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl Acad. Sci. USA* **90**, 6444–6448.
8. Verma, R., Boleti, E., and George, A. J. (1998) Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. *J. Immunol. Methods* **216**, 165–181.
9. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res.* **19**, 4133–4137.
10. Carter, P., Kelley, R. F., Rodrigues, M. L., Snedecor, B., Covarrubias, M., Velligan, M. D., et al. (1992) High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Biotechnology* **10**, 163–167.
11. Knappik, A. and Pluckthun, A. (1995) Engineered turns of a recombinant antibody improve its in vivo folding. *Protein Engineering* **8**, 81–89.
12. Coia, G., Ayres, A., Lilley, G. G., Hudson, P. J., and Irving, R. A. (1997) Use of mutator cells as a means for increasing production levels of a recombinant antibody directed against hepatitis B. *Gene* **201**, 203–209.
13. Hollenberg, C. P. and Gelissen, G. (1997) Production of recombinant proteins by methylotrophic yeasts. *Curr. Opin. Biotechnol.* **8**, 554–560.
14. Sreekrishna, K., Brankamp, R. G., Kropp, K. E., Blankenship, D. T., Tsay, J. T., Smith, P. L., et al. (1997) Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* **190**, 55–62.
15. Luo, D., Mah, N., Krantz, M., Wishart, D., Jacobs, F., and Martin, L. (1997) High-level secretion of single-chain antibody in *Pichia* expression system. *Biotechnol. Tech.* **11**, 759–761.
16. Harrison, J. L., Williams, S. C., Winter, G., and Nissim, A. (1996) Screening of phage antibody libraries. *Methods Enzymol.* **267**, 83–109.
17. Bruggeman, M. and Taussig, M. J. (1997) Production of human antibody repertoires in transgenic mice. *Curr. Opin. Biotechnol.* **8**, 503–508.
18. Tomlinson, I. and Holliger, P. (2000) Methods for generating multivalent and bispecific antibody fragments. *Methods Enzymol.* **326**, 461–479.

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